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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,274,414**, on June 11, 1999, by **UNIVERSITE LAVAL**, assignee of Hélène Jacques, Charles Lavigne and André Marette, for "Dietary Fish Protein for Use in Restoring Normal Insulin Function Insulin-Resistant Individuals".

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ABSTRACT

The present invention describes the use of fish protein, namely cod prot in, to improve the peripheral insulin resistance in a mammal. Fish protein has been administered to rats submitted to high sucrose or high fat diets, which are animal models for diabetes. It has been found that fish protein efficiently controls glucose utilization, particularly in muscle tissue. This effect is not observed wh n rats are given casein or soy proteins. Fish protein is therefore promising for controlling insulin resistance, diabetes and obesity complications.

TITLE OF THE INVENTION

Dietary Fish Protein for Use in Restoring Normal Insulin Function Insulin-Resistant Individuals

FIELD OF THE INVENTION

This invention relates to compositions comprising fish proteins used for preventing insulin resistance or restoring normal insulin function in insulin-resistant subjects, thereby useful for the prevention of the treatment of diabetes type II.

BACKGROUND OF THE INVENTION

Glucose intolerance is a preclinical syndrome associated with the development of non-insulin-dependent diabetes mellitus (NIDDM), which is characterized by an abnormally low response of the target cells for insulin-mediated glucose utilization, inducing high plasma insulin levels [1] and hypertriglyceridemia [2].

Several studies have demonstrated that the macronutrient composition of the diet is an important determinant of the quality of the insulin action. Although most studies have examined the role of high-fat [3-8]), low-soluble fiber [9] or high-sucrose diets [10] on the impairment of insulin action, relatively few studies focused on the impact of dietary proteins. Up to now, a high-protein intake (60% of energy) has been shown to impair glucose metabolism [11] but little information is available concerning the role of dietary protein source. In this respect, when compared with casein, soy protein has been shown to decrease serum insulin concentrations in fasted normoglycemic rats [12]. Moreover, studies in rats [13], and in humans [14] showed a short-term reduced postprandial insulin response to a single test meal containing soy protein compared with casein. Iritani et al. [15] showed that dietary soy protein in a saturated high-fat diet may help to improve insulin sensitivity by increasing insulin mRNA levels in liver and adipose tissues. Interestingly, cod protein has been also shown to reduce plasma glucose compared with casein in normoglycemic rats in the fasting state [16]. However, little is known on the effects of cod and soy proteins on glucose tolerance, and on the postprandial plasma glucose and insulin response to a meal in rats maintained on controlled diets for a long-term period.

Differences in dietary protein structure and plasma amino acid profiles have been proposed as the mechanism explaining for protein-dependent modifications in glucose and insulin dynamics [17] [18]. According to Sanchez and Hubbard [18], the lysine/arginine ratio in the dietary proteins was related to plasma insulin concentrations. Plasma branched-chain amino acids, particularly leucine, can also influence carbohydrate metabolism by decreasing glucose oxidation by the skeletal muscles [19, 20].

There is therefore an interest in verifying if prolonged feeding either cod or soy

protein compared with casein in controlled diets can exert a beneficial effect on glucose tolerance and on the postprandial plasma glucose and insulin respons s.

It is well documented that the incidence of diabetes is low in populations consuming large amounts of fish and marine foods such as Greenland and Alaskan Eskimos and Alaskan Indians (36, 37). Apparently, these findings could not be explained by differences in genetic predisposition or obesity (37). These effects have been primary attributed to the presence of fish oil in fish. Storlien et al (38) observed increased insulin sensitivity in liver tissue and skeletal muscle in experiments conducted in rats when dietary n-6 fatty acids were partially replaced by fish oil. Beneficial effects of fish oil on insulin sensitivity in diabetic patients have been documented by Popp-Snijders et al (39). However, short-term experiments on the effect of fish oil on glucose homeostasis in diabetic patients have not been conclusive so far. In a recent meta-analysis (40), the consumption of 3 g/day of n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), had no effect on glycemia in NIDDM but resulted in a significant decrease in mean serum triglycerides levels. The results of this meta-analysis in addition demonstrated a dose-response relationship. In NIDDM subjects, for every increase in EPA dose of 1 g/day, LDL cholesterol and glycosylated hemoglobin concentrations significantly increased and for every increase in DHA dose of 1 g/day, fasting glucose and glycosylated hemoglobin concentrations significantly increased. Earlier studies have previously shown that the addition of n-3 fatty acids could increase glucose and glycosylated hemoglobin levels to some extent (41-43). Thus, doubt remains on the net effect of fish oil intake on glycemic control in diabetes. On the other hand, Feskens et al (44) sustained that the usual fish consumption was inversely associated to the incidence of glucose intolerance and type II diabetes. Individuals who consumed fish had a much lower incidence of glucose intolerance and type II diabetes than those who did not consume any (25% vs 47%). The subjects consumed small quantities of fish (24 g/d) mainly lean and providing little doses of n-3 fatty acids (140 mg) daily. These authors proposed that some other constituent in fish could improve glucose metabolism.

If we prove that the protein moiety of either soy, fish or casein has an effect on plasma glucose and insulin curve responses following an intravenous glucose tolerance test (IVGTT) and a test meal and find that dietary protein induced a lower insulin response to the glucose load, this would suggest an improved peripheral insulin sensitivity. Therefore there is an interest to investigate whether fish and soy proteins, as compared with casein protein, could improve whole-body glucose insulin action and peripheral glucose uptakes in high-sucrose or high-fat fed rats, well established models of insulin resistance.

SUMMARY OF THE INVENTION

We aim at determining the effects of various dietary proteins on plasma glucose and insulin curve responses following an intravenous glucose tolerance test (IVGTT) and after a test meal in rats. For 28 days male Wistar rats were fed isoenergetic high-sucrose diets containing either casein, soy protein or cod protein, and providing (w/w) 20% protein, 10% coconut oil, 1% corn oil, 59% sucrose and 1% cholesterol. In the fasting state, cod protein- and soy protein-fed rats had lower plasma glucose and insulin concentrations, compared to casein-fed animals. Plasma glucose response after intravenous glucose bolus was lower after 10 and 20 minutes in cod protein- and soy protein-fed rats than in casein-fed rats, resulting in lower incremental areas under glucose curves and in a higher rate of glucose disappearance (Rd) with cod protein than with casein. Cod protein induced a lower insulin response to the glucose load particularly during the late-phase insulin secretion (10 to 50 minutes), suggesting an improved peripheral insulin sensitivity in comparison with casein. In the test meal experiment, after a 12-hour fast, each dietary group received 5 g of their usual purified diet during 30 minutes. In the postprandial state, plasma glucose responses were similar regardless of protein origin. Postprandial plasma insulin, C-peptide and triglyceride concentrations were lower in cod protein- and soy protein-fed rats than in casein-fed rats at several time points following the test meal. Higher postprandial plasma arginine concentrations as well as lower branched-chain or essential amino acids could be involved in the improvement of insulin sensitivity in cod and soy protein-fed rats. In conclusion, the metabolic response to three common dietary proteins indicate that soy protein and cod protein when compared to casein, improved insulin sensitivity and reduce fasting and postprandial plasma insulin response in rats fed high-sucrose diets.

Since we have shown that rats fed a saturated high-fat diet develop peripheral insulin resistance. We have examined the effects of feeding casein, cod or soy proteins on whole-body insulin action and peripheral tissues glucose utilization in high-fat fed rats. High-fat feeding in casein- or soy protein-fed rats led to severe insulin resistance (glucose infusion rates (GIR) : 12.2 ± 1.1 and 7.9 ± 1.1 $mg \cdot kg^{-1} \cdot min^{-1}$ respectively) as compared to chow-fed (non high-fat) controls (16.9 ± 2.0 $mg \cdot kg^{-1} \cdot min^{-1}$). In marked contrast, cod feeding completely prevent high-fat-induced peripheral insulin resistance (GIR: 19.7 ± 2.7 $mg \cdot kg^{-1} \cdot min^{-1}$, $p<0.05$ compared to casein- or soy-fed rats). Measurements of 2-[^{3}H]-deoxy-D-Glucose (2-DG) uptake in individual tissues revealed that cod-fed rats had greater rates of insulin-stimulated 2-DG uptakes in skeletal muscles (all fiber types), heart, brown adipose tissue, but not in white adipose tissues. Basal 2-DG uptake was not different among experimental groups. In conclusion, feeding cod protein, but not casein or soy proteins, completely prevented the development of insulin resistance in high-fat fed rats. Interestingly, the beneficial

effects of cod protein was specific for energy consuming tissues such as skeletal muscle and brown adipose tissue, and occurred without any changes in body weight gain or adipose tissue accretion, suggesting that fish protein prevents obesity-linked insulin resistance. We investigated the benefits of fish protein in diets, for improving glucose utilization in high-fat fed rats.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The objects, advantages and other features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

Although the present invention has been described hereinabove by way of a preferred embodiment thereof, this embodiment can be modified at will, within the scope of the appended claims, without departing from the spirit and nature of the subject invention.

Figure 1. A) Changes in plasma glucose concentrations in the fasted state and after i.v. glucose bolus in rats fed either the casein, cod protein diet or soy protein for 28 days. **(B)** Glucose area response to IVGTT in arbitrary units. **(C)** Rate of glucose disappearance (Rd). **(D)** Changes in plasma insulin concentrations in the fasted state and after i.v. glucose bolus in rats fed either the casein, soy protein or cod protein diet. **(E)** Insulin area responses to IVGTT in arbitrary units. Groups bearing different letters for a given time point are significantly different ($P<0.05$). Areas and Rd are significantly different ($p<0.05$) if they do not share a common letter. Values are means ± SEM.

Figure 2A). Glucose disposal rate (GDR) to maintain euglycemia during steady-state (60-120 minutes) insulin infusion in fasted state. **B)** Plasma 2-deoxy-D-glucose disappearance (Kp). Rats were fed either casein, cod or soy protein diets during 28 days. Bars represent means ± SEM of data obtained from 3 to 4 rats in each group. Groups without common letter differ at $p<0.05$.

Figure 3. A) Changes in plasma glucose concentrations in the fasted state and after the test meal in rats fed either the casein, cod protein diet or soy protein for 28 days. Meals consisted of approximately 5 g of their current diet. **(B)** Glucose area response to the test meal in arbitrary units. **(C)** Changes in plasma insulin concentrations in the fasted state and the test meal in rats fed either the casein, soy protein or cod protein diet. **(D)** Insulin area responses to the test meal in arbitrary units. Groups bearing different letters for a given time point are significantly different ($p<0.05$). Areas are significantly different ($p<0.05$) if they do not share a common letter. Values are means ± SEM.

Figure 4 (A). Changes in plasma C-peptide concentrations in the fasted state and after the test meal in rats fed either the casein, soy protein or cod protein diet for 28 days. **(B)** Plasma C-peptide area response to the test meal in arbitrary units. **(C)** Changes in plasma glucagon concentrations in the fasted state and after the test meal in rats fed either the casein, soy protein or cod protein diet. **(D)** Plasma glucagon area response to the test meal in arbitrary units. Groups bearing different letters for a given time point are significantly different ($p<0.05$). Areas are significantly different ($p<0.05$) if they do not share a common letter. Values are means ± SEM.

Figure 5 (A). Changes in plasma insulin/glucagon ratio concentrations in the fasted state and after the test meal in rats fed either the casein, soy protein or cod protein diet for 28 days. **(B)** Changes in plasma triglyceride concentrations in the fasted state and after the test meal in rats fed either the casein, soy protein or cod protein diet. Groups bearing different letters for a given time point are significantly different ($P<0.05$). Values are means ± SEM.

Figure 6 (A). Changes in plasma free amino acids 30 minutes after the test meal in rats fed either the casein, soy protein or cod protein diet for 28 days. **(B)** Changes in plasma free amino acids 120 minutes after the test meal in rats fed either the casein, soy protein or cod protein diet for 28 days. Groups bearing different letters are significantly different ($P<0.05$). Values are means ± SEM.

Figure 7. Glucose infusion rate (GIR_{60-120}) to maintain euglycemia during steady-state (60-120 min) insulin infusion in fasted rats. Rats were fed either casein, cod, or soy proteins with the high-fat diets during 4 weeks. Chow-fed rats value (dotted line) indicate normal GIR_{60-120} value. Values are means ± SE for 7 to 9 in each group. Groups without common letter differ at $P<0.05$.

Figure 8. *In vivo* 2-deoxy-D-glucose uptake in white tibialis, white gastrocnemius and quadricep muscles during basal and hyperinsulinemic-euglycemic clamps. The mean value for chow-fed rats is represented by a dotted line, indicate normal *in vivo* 2-deoxy-D-glucose uptake value in hyperinsulinemic-euglycemic clamp and was not statistically different as compared with high fat-fed rats fed cod protein. Bars represent means ± SEM of data obtained from 7 to 9 rats. Groups without common letter differ at $P<0.05$.

Figure 9. *in vivo* 2-deoxy-D-glucose uptake in soleus, red tibialis, red gastrocnemius and EDL muscles during basal and hyperinsulinemic-euglycemic clamps. The mean value for chow-fed rats is represented by a dotted line. Bars represent means ± SEM of data obtained from 7 to 9 rats. Groups without common letter differ at $P<0.05$.

Figure 10. *In vivo* 2-deoxy-D-glucose uptake in heart and interscapular brown adipose tissue (BAT) during basal and hyperinsulinemic-euglycemic clamps. The mean value for chow-fed rats is represented by a dotted line. Bars represent means ± SEM of data obtained from 7 to 9 rats. Groups without common letter differ at $P<0.05$.

Figur 11. *In vivo* 2-deoxy-D-glucose uptake in white gonadal (Wgona) and white

retroperitoneal (WRetro) fat pad tissues during basal and hyperinsulinemic-euglycemic clamps. The mean value for chow-fed rats is represented by a dotted line. Bars represent means ± SEM of data obtained from 7 to 9 rats. Groups without common lett r differ at P<0.05.

Figur 12. *In vivo* 2-deoxy-D-glucose uptake in brain during basal and hyperinsulinemic-euglycemic clamps. The mean value for chow-fed rats is represented by a dotted line. Bars represent means ± SEM of data obtained from 7 to 9 rats. Groups in without common letter differ at P<0.05.

EXAMPLE 1:

We investigated the effects of casein, cod and soy proteins on plasma glucose and insulin curves responses after 1) an intravenous glucose tolerance test (IVGTT) and 2) a test meal containing either one of these proteins, in rats maintained on controlled diets of varying protein for 4 weeks. Physiological curve responses of plasma C-peptide, glucagon and triglycerides were also determined following the test meal. To gain insight this protein-dependent mechanism, fasted and postprandial plasma amino acid concentration changes were performed and coefficient of correlation were calculated between plasma amino acid changes and plasma glucose, insulin, C-peptide and glucagon concentrations.

MATERIALS AND METHODS

Animals

Sixty male Wistar rats (Charles River, St. Constant, Qc, Canada) weighing approximately 240 g on arrival were individually housed in wired-mesh cages in a temperature- and humidity-controlled room with a daily dephased 12-h light/dark cycle (lights on at 2200 to 1000h). Upon arrival, all rats were fed a grounded nonpurified commercial diet (Purina rat chow, Ralston Purina Inc., Lasalle, Qc, Canada) for at least 6 days. At the end of this baseline period, rats were divided into three groups of same average weights. Purified diets and tap water were provided *ad libitum* for 28 days. Food intake was estimated every day by subtracting the food spillage weight from the initial food weight, and body weight was measured weekly. The animal facilities meet the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Animal Care Committee of Laval University.

Diets

After the baseline period, all animals were assigned to one of the three purified powdered diets varying in protein source, namely casein, cod protein or soy protein and were fed for 28 days. The composition of each purified diet is detailed in Table 1. All diet ingredients, except vitamin mix (Teklad, Madison, WI) and cod protein, were purchased from ICN, Cleveland, OH. Cod protein was prepared in our laboratory as follows: frozen cod fillets were lyophilized and subsequently submitted to a 24-h delipidation using diethylether as solvent in an Soxlet-type apparatus (Canadawide

Scientific, Montréal, Qc, Canada). Cod protein was then dried to evaporate remaining solvent , befor grinding it to a fine powder. Cod protein contained 92% protein and was virtually free of residual lipids (0.1%). Ingredients for the purified diets were mixed and stored at -20 °C until used. The amino acid composition of these proteins was measured by an ion-exchange chromatography method using Beckman amino acid analyzer (Palo Alto, CA) model 6300. Data are expressed as g/100g of amino acids and are given in Table 2. The energy content of the diets was measured with an automatic adiabatic calorimeter (Model 1241; Parr Instruments, Moline, IL, USA). Diets were found to be isoenergetic: casein (19.91 kJ/g), soy protein (19.95 kJ/g), cod protein (19.66 kJ/g). The protein content (N x 6.25) was determined with Kjeldahl Foss autoanalyser (Model 1612; Foss Co., Hillerod, Denmark). The level of protein in the purified diets was adjusted to an isonitrogenous basis at the expense of carbohydrates

Experimental Protocols

At day 25, all rats were cannulated via the jugular vein under isoflurane anesthesia. Food intake was near normal postoperatively, and all rats were within 4 % of surgery weight on the day of the study. Blood sampling were carried out in a 15 x 30 cm open plastic box to which rats were accustomed and in which they remained undisturbed during the experiment. Experiments 1 and 2 were evaluated in separate groups of animals.

Experiment 1: Intravenous Glucose Tolerance Test (IVGTT)

At day 28, after a 12-h fast, 10 rats per dietary group were injected with 1.5 $mL \cdot kg^{-1}$ of body weight of a 35 % glucose solution dissolved in saline as a bolus via the jugular catheter. The catheter was then flushed with saline. Blood samples (300 μL) were drawn through the catheter with EDTA-containing syringes (1.5 $g \cdot L^{-1}$ blood) before (0 minute) and 2, 5, 10, 20, and 50 minutes after the glucose load and were stored on ice. The plasma was separated by centrifugation and stored at -80°C until analysis. All erythrocytes were pooled, resuspended in saline and injected into the animals after the 20 and 50 minute samples.

Experiment 2: Test Meal

The same experimental diets and jugular canulation protocols as described above was conducted. At day 28, after a 12-h fast, and at the beginning of the dark period, 10 rats per dietary group received 5 g of their assigned experimental diet for 30 minutes. After that time any uningested food was removed. Blood samples were obtained before the beginning of the test meal (-30 minutes) and at 0 (end of the meal), 30, 60, 120 and 240 minutes. Because rats provided limited amounts of blood volumes, measurements of plasma C-peptide, glucagon and amino acids were performed at -30, 30 and 120 minutes only. The plasma was separated by centrifugation and stored at -80°C until analysis. All erythrocytes were pooled, resuspended in saline and reinjected into the animals after the 30 and 120 minut s

samples.

Analytical Methods

Plasma glucose l v ls were analyzed using a glucose oxidase method (YSI 2700 Select, Yellow Springs, OH) and plasma insulin, C-peptide and glucagon levels wer measured with a RIA-method (Linco Research, St. Charles, MO) using rat insulin, C-peptide and glucagon standards. Triglycerides were assayed by an enzymatic method using a reagent kit from Boehringer Mannheim (Montréal, Qc, Canada). Amino acid composition of plasma samples after deproteinization, as reported by Galibois et al [21]], were analysed by an ion-exchange chromatography method using Beckman amino acid analyzer (Palo Alto, CA) model 6300, and data are expressed as μmol/L. Incremental areas under the curves obtained during IVGTT and Test meals were calculated with a computer graphic program, using 0 or -30 minutes as baseline values for IVGTT and test meal respectively. Rates of glucose disappearance (Rd), expressed as percent disappearence per minute, were calculated from the slope of the regression line obtained with the logarithm transformed plasma glucose value between 2 and 20 minutes after glucose administration.

Statistical Analysis

Data were analysed with the general linear model program (GLM) on the SAS statistical package for personal computers. Data obtained from serial sampling were analyzed using ANOVA with repeated measures, with time as the repeated variable. Comparisons between protein groups were done using the Duncan multiple range test. Differences were considered significant at $p<0.05$. The relationships between hormonal parameters and free amino acids were determined by the calculation of Pearson correlation coefficients [22]. All results are presented as mean ± SEM.

RESULTS

Table 2 indicates the amino acid composition of the tested dietary proteins. Casein showed the highest value for proline, tyrosine and valine while cod protein contained more alanine and lysine. Notably, the arginine level was 2 times more important in soy protein and cod protein than in casein. The glycine and aspartic acid levels were also higher in soy protein and cod protein than in casein. The total amount of branched-chain amino acids was slightly higher in casein than in cod protein and soy protein, while the sum of essential amino acids was slightly higher in casein and cod protein than in soy protein. The lysine/arginine ratio was higher in casein (2.4) than in soy protein (0.9) and cod protein (1.5).

After 28 days of treatment, rats displayed comparable daily food intake and body weight gain regardless the source of dietary protein (Table 3). The food intake for the last meal, (experiment 2: Test meal) was similar between the protein groups.

Plasma glucose and insulin responses, incremental areas under the glucose and insulin curves and rates of glucose disappearance (Rd) during IVGTT are shown

in Fig. 1. After 4-week feeding, fasting plasma glucose (0 minute) was lower in cod protein and soy protein-fed rats than in casein-fed rats (11 % and 10 % respectively, $p<0.05$) (Fig. 1 A). Cod and soy protein diets resulted in significant ($p<0.05$) lower plasma glucose 10 and 20 minutes after intravenous glucose load as compared to casein diet (Fig.1A). Cod (24%, $p<0.05$) and soy (22%, $p<0.05$) proteins induced smaller incremental areas under the glucose curves than casein (Fig. 1B). Cod protein-fed rats induced significantly ($p<0.05$) higher Rd than casein (22%, $p<0.05$). Rd was intermediate in soy protein-fed rats (Fig. 1C).

Fasting plasma insulin (0 minute) was lower in cod protein- and soy protein-fed rats than in casein-fed rats (45 % and 40 % respectively, $p<0.05$) (Fig. 1D). Furthermore, cod protein, when compared with casein, induced a lower insulin response ($p<0.05$) to the glucose load related for the most part to the late-phase insulin secretion (10-50 minutes) (Fig. 1D). Nevertheless, the incremental areas under the insulin curves were similar between protein groups (Fig. 1E).

To determine whether the greater insulin sensitivity of cod protein- and soy protein-fed rats is due to an increased peripheral insulin action, whole-body insulin action and peripheral tissue glucose utilization have been measured by euglycemic hyperinsulinemic clamp technique. Cod protein-and soy protein-fed rats displayed increased glucose disposal rate (GDR) as compared to rats fed casein (Fig. 2A). Plasma 2-deoxyglucose disappearance rate (Kp) was also higher in cod protein- and soy protein-fed rats than in casein-fed rats (Fig. 2B). In accordance with an increased peripheral action of insulin in cod protein-and soy protein-fed rats, higher insulin-stimulated glucose uptake was observed in red gastrocnemius muscle of these rats (472 ± 26 nmol/min/g and 412 ± 18 nmol/min/g, respectively) as compared to casein-fed animals (213 ± 24 nmol/min/g; $p<0.05$).

Fig. 3 shows fasting and postprandial plasma glucose and insulinconcentrations and incremental areas under the glucose and insulin curves of plasma collected before and after the test meal in animals fed the diets for 4 weeks. Fasting plasma glucose concentrations (-30 minutes) were significantly ($p<0.05$) lower in cod protein- (10 %, $p<0.05$) and soy protein-fed rats (9 %, $p<0.05$) than in casein-fed rats (Fig. 3A). Postprandial plasma glucose reach a peak responses after 1 hour (Fig. 3A) and the incremental areas under the glucose curves after the test meal (Fig. 3B) were similar regardless of the protein consumed.

Plasma insulin responses and the incremental areas under the insulin curves during the test meal are shown in Fig. 3C and 3D respectively. In the fasting state (-30 minutes), lower ($p<0.05$) plasma insulin concentrations were observed in cod protein- (61%) and soy protein-fed (56%) rats than in casein-fed rats. Postprandial plasma insulin concentrations were lower ($p<0.05$) in soy protein-fed rats than in casein-fed rats at several time points (30, 60 and 120 minutes) following the test meal.

Postprandial plasma insulin concentrations were lower ($p<0.05$) in cod protein-fed rats compared with casein-fed rats immediately after the test meal (0 minute) and 30 and 60 minutes after the test meal (Fig. 3C). The incremental areas under the insulin curve were lower ($p<0.05$) with cod protein (25%) and soy protein (35%) than with casein (Fig. 3D).

Th plasma C-peptide curve response is illustrated in Fig 4A. In the fasting state (-30 minutes), C-peptide concentrations were lower in rats fed soy (23%) and cod proteins (30%) compared with rats fed casein. In the postprandial state, plasma C-peptide concentrations were lower (28%) at both 30 and 120 minutes following the test meal in soy protein- compared with casein-fed rats. Plasma C-peptide concentrations were intermediate at these two time points in cod protein-fed rats. The incremental area under C-peptide curves were similar whatever the dietary protein consumed.

Plasma levels of the counterregulatory hormone glucagon is shown in Fig 4C. Glucagon concentrations were comparable among protein groups in fasted rats after 4 weeks of treatment. Following the ingestion of cod and soy protein meals there was no increase in plasma glucagon concentrations. Casein induced a glucagon concentration peak after 30 minutes which is 26% ($p<0.05$) higher than that soy protein. Two hours after the test meal, the glucagonic response was lower in cod protein- and soy protein-fed rats than casein-fed rats, by 20 and 25% respectively, $p<0.05$. The incremental area under the glucagon curve was greater with casein than with soy protein by 58 % ($p<0.05$) (Fig. 4D). It is also important to note that the insulin/glucagon ratio was significantly ($p<0.05$) higher in rats fed casein than in those fed cod or soy proteins before and 30 minutes after the test meal (Fig. 5A).

Plasma triglyceride responses are shown in Fig. 5B. In the fasting state, soy protein- and cod protein-fed rats had lower (25 % and 30 % respectively, $p<0.05$) plasma triglyceride concentrations compared to casein-fed rats. In the postprandial state, plasma triglyceride concentrations were lower in soy protein- and cod protein-fed rats (40 % and 25 % respectively, $p<0.05$) than in casein-fed rats 120 minutes after the test meal, whereas at 240 minutes after the test meal, plasma triglycerides were lower only in soy protein-fed rats compared with casein-fed rats (37 %, $p<0.05$).

To gain insight the mechanism whereby glucose metabolism was affected by dietary protein, measurements of plasma amino acid levels were performed. Fasting plasma amino acid levels are presented in Table 4. In the fasting state, plasma L-aspartic acid and L-glycine concentrations of the soy protein-fed rats were higher than those of cod protein- and casein-fed rats. Plasma L-citrulline concentrations were higher with casein than with cod and soy proteins. Plasma L-histidine concentrations of cod protein-fed animals were lower than those of casein-fed and soy protein-fed animals. Plasma L-taurine concentrations were significantly higher in rats fed cod protein than in those fed casein, which were nevertheless higher than in those fed soy

protein. However, L-arginine, L-lysine, lysine/arginine ratio as well as the plasma sum of total and total branched and essential amino acid concentrations were similar between dietary groups. Interestingly, plasma L-arginine was negatively correlated with fasting plasma C-peptide concentrations (r=-0.42, p=0.04, n=24) confirming the antisecretagogue role of plasma arginine in physiological concentration. No other fasted amino acid levels was correlated with measured parameters.

Only significant changes in plasma amino acid concentrations after the test meal (30 and 120 minutes vs fasted -30 minutes) are illustrated in Fig. 6A and 6B respectively. Changes in postprandial L-alanine (30 minutes), L-tyrosine (30 minutes), L-leucine (30 minutes), L-proline, and L-valine (30 and 120 minutes) were greater in rats fed casein than those in rats fed cod or soy protein. Changes in postprandial plasma L-arginine (30 and 120 minutes) resulted in lower concentrations in casein-fed rats compared with those in cod and soy protein-fed rats. Thus, changes in L-methionine (30 and 120 minutes) L-alanine and L-lysine (120 minutes) after the test meal were larger with casein- and cod protein-fed rats compared with those obtained in soy protein-fed rats. Postprandial plasma L-taurine changes (30 minutes) were higher in cod protein-fed rats whereas those in soy protein-fed rats, but casein-fed rats induced intermediate changes. After 120 minutes, L-taurine changes were significantly higher in cod protein group compared to casein and soy proteins groups. Thirty (30) and 120 minutes after the meal, sum of branched free amino acids were higher in casein fed group (30 minutes: 515 ± 48 μmol/L, $p<0.05$; 120 minutes: 492 ± 60 μmol/L, $p<0.05$) compared to cod (30 minutes: 375 ± 33 μmol/L; 120 minutes: 385 ± 24 μmol/L) and soy protein (30 minutes: 393 ± 25 μmol/L; 120 minutes: 393 ± 33 μmol/L). Postprandial sum of essential free amino acids were higher in casein fed group (30 minutes: 1552 ± 122 μmol/L, $p<0.05$; 120 minutes: 1487 ± 121 μmol/L, $p<0.05$) compared to cod (30 minutes: 1255 ± 97 μmol/L; 120 minutes: 1155 ± 71 μmol/L) and soy protein (30 minutes: 1207 ± 57 μmol/L; 120 minutes: 1160 ± 71 μmol/L). Postprandial plasma L-alanine was correlated with posprandial plasma glucagon both after 30 (r=0.63, p=0.0005, n=26) and 120 minutes (r=0.46, p=0.02, n=23) confirming the glucagon secretagogue role of plasma alanine in physiological concentration. Branched free amino acids after 120 minutes were also correlated with postprandial insulin after 120 minutes (r=0.56, p=0.0061, n=22).

DISCUSSION

In the present studies, three diets, differing only in their sources of dietary proteins were used to fed rats. Indexes of glucose tolerance were evaluated with IVGTT, and following a test meal. After 28 days, the casein diet showed a deterioration in the indices of insulin sensitivity relative to cod and soy protein diets and developed fasting and postprandial hyperinsulinemia, and hypertriglyceridemia. Our data on fasted and postprandial plasma amino acid profile supports the regulatory role

of amino acids in dietary proteins, on the improvement of glucose metabolism.

In the present study, rats fed cod and soy protein diets showed lower fasting plasma glucose concentrations than those fed casein, as previously observed in our laboratory [16]. Fasting plasma insulin levels were also significantly lower in rats fed cod and soy protein diets, agreeing with data reported by Vahouny et al. [12] who showed reduced serum insulin concentrations in fasted soy protein-fed rats than in casein-fed rats. We also found lower plasma glucose responses and incremental areas under glucose curves in soy protein- and cod protein-fed rats as well as lower plasma insulin responses in cod protein-fed rats following the glucose load.

Our additional data on postprandial plasma insulin concentrations following the test meal are in good accordance with those of Hubbard and Sanchez [14] who reported lower blood insulin levels in humans fed a soy protein meal versus a casein meal, and with our hypothesis that soy protein and cod protein, compared with casein, may improve the physiological postprandial curve response of plasma insulin in rats. The abnormal glucose intolerance observed in casein animals suggest either after the IVGTT and the test meal that insulin was less efficient in stimulating glucose disposal in casein-fed rats compared to cod protein- or soy protein-fed rats.

Fasting and postprandial plasma insulin responses to dietary proteins were most likely consequent to pancreatic insulin secretion of rats fed different dietary proteins, as demonstrated by plasma C-peptide curves which are highly correlated with fasted ($r=0.83$; $p=0.001$, $n=24$) and postprandial insulin curves (30 minutes: $r=0.87$, $p=0.0001$, $n=24$; 120 minutes: $r=0.77$, $p=0.0001$, $n=24$). Both fasting and postprandial C-peptide responses suggest a decreased pancreatic insulin secretion and an improved glucose tolerance with cod and soy proteins compared with casein.

Glucose tolerance is promoted by two simultaneously ongoing processes: 1) suppression of gluconeogenesis and hepatic glucose production and 2) stimulation of peripheral glucose uptake. In the present study, measurements of plasma glucagon, which is gluconeogenic, indicated lower glucagon secretion with cod and soy proteins as compared to casein in the postprandial state, and lower plasma insulin/glucagon ratios with cod and soy proteins as compared to casein both in the fasting state and 30 minutes after the test meal. These results indicate a suppressive effect of cod and soy proteins on plasma glucagon secretion suggesting a reduction of gluconeogenesis. On the other hand, peripheral insulin sensitivity may be explained by higher rates of insulin-stimulated glucose transport in skeletal muscles, the main site of glucose disposal in the post-absorptive state [23].

Several links between hypertriglyceridemia and insulin resistance may be invoked as possible causes for their common occurrence. Insulin influence both the rate of hepatic triglyceride secretion into the circulation and the rate of disappearance from the blood stream through its action on lipoprotein lipase (LPL) activity [24] In the

present study, fasting and postprandial triglycerides were lower in rats fed either cod or soy protein than in those fed casein. Lower fasting and postprandial insulin mia provoked by either cod or soy protein could have decreased tissue fatty acid mobilization and in turn the synthesis and secretion of VLDL triglycerides in the liver of cod- and soy protein-fed rats [25], reducing their triglyceridemia when compared with casein-fed rats. The present findings thus suggest that the improvement in both fasting and postprandial triglyceridemia with cod and soy proteins is related to an enhanced insulin action.

A number of scientific reports [26], [27], [28], [29], [30] have emphasized the role of amino acids as mediators of plasma glucose and insulin responses. Indeed, at levels found in dietary proteins, arginine has been associated with a decrease of fasting insulin [17] contrary to pharmacological doses of arginine which have been associated with an increase of both insulin and glucagon concentrations [26, 31]. Mulloy et al. [32] demonstrated that a diet containing 1.0% arginine, which is an amount close to what is found in the cod protein (1.2%) and soy protein (1.5%) diets, induced lower plasma insulin concentrations 30 and 45 minutes following a IVGTT than a diet containing 0.5% arginine, which is closely equivalent to the arginine level found in our casein diet (0.6%). Furthermore, Vahouny et al. [12] showed that the addition of arginine to a casein diet, to simulate the lysine/arginine ratio of soy protein, resulted in serum fasting insulin levels similar to those measured in rats given a soy protein diet. In the present study, changes in postprandial plasma L-arginine resulted in higher concentrations in rats fed cod protein and soy protein than in those fed casein. This can likely attributed to the higher arginine content of native soy and cod protein compared to casein. In the present study, negative correlations were found between plasma fasting arginine and C-peptide concentrations ($r=-0.42$, $p=0.04$, $n=26$) confirming the antisecretagogue role of physiological levels of plasma arginine on plasma insulin. Thus, the present results suggest that the higher arginine content of cod and soy proteins, compared to that of casein, can induce higher postprandial plasma arginine concentrations that could be associated with lower pancreatic postprandial insulin secretions and chronic reductions in insulin concentrations in the fasting state. The present data also indicate that both cod and soy proteins induced lower postprandial changes in plasma L-leucine, L-proline and L-valine concentrations 30 and 120 minutes after the test meal compared with casein. Their lower content in the correspondent dietary proteins are likely at the origin of these differences. Postprandial plasma L-alanine responded similarly as leucine and valine did 30 minutes following the test meal because it derived mainly from branched-chain amino acids deamination [33]. Being reported to be one of the most potent glucagon secretagogue among the amino acids [29], the higher increase of plasma L-alanine 30 minutes following the test meal could have promoted glucagon secretion 30 minutes

after the test meal in casein fed rats. The positive correlations between postprandial plasma L-alanine and glucagon levels observed in the pres nt study support this physiological role. Sums of plasma concentrations of free branched-chain (leucine, valine and isoleucine) and free ess ntial amino acids were low r in cod and soy protein groups than in the casein group after the test meal whereas these values were nonsignificantly different in the fasting state. These results are in good accordance with those of Fajans et al. [26] who reported that infusion of the essential amino acids induced an increase in plasma insulin levels. These findings thus suggest that lower increases in the postprandial plasma leucine and valine, or total branched-chain or essential amino acids, following the consumption of either cod or soy proteins could provoke a decrease in postprandial plasma insulin concentrations. Fasted plasma total free and branched free amino acids are similar whatever the dietary group. However, in postprandial state they were greatest with casein compared with cod and soy proteins. Thus the insulin response may be related to the rate of digestion of proteins.

Two mechanisms have been proposed to explain the role of amino acids in controlling insulin action. An early report [26] have suggested that leucine and/or other essential amino acids, either individually or in combination, stimulate the pancreatic release of insulin. More recent reports [28, 34] have hypothesized that amino acids can diminish insulin's ability to stimulate peripheral glucose transport inducing a loss of cellular insulin sensitivity. Indeed, infusion of branched chain amino acids, which are predominantly metabolized in skeletal muscles, has been shown to inhibit insulin-mediated glucose uptake in the forearm muscle [35]. However, the mechanism by which dietary protein induce glucose intolerance at the cellular level is not yet well understood. Patti et al. [34] have proposed that some amino acids inhibit critical early steps in postreceptor insulin action for glucose transport. There is thus a possibility that higher postprandial plasma arginine concentrations as well as lower branched-chain amino acids or lower essential amino acids as observed in cod and soy protein-fed rats compared with casein fed rats can stimulate glucose transport at the cellular level, resulting in decreased insulin secretion by pancreatic cells as a response to a reduction of plasma glucose. Further studies are nevertheless required to elucidate the mechanism linking these amino acids to the biological action of insulin and to identify their metabolic pathways mediating insulin sensitivity of the peripheral glucose transport system.

The results of the present study indicate that when compared with casein, soy protein and cod protein reduce fasting and postprandial plasma insulin response in rats. These data thus suggest that soy protein and cod protein fed rats have improved insulin sensitivity for glucose disposal as compared to casein-fed rats. We have also identified some amino acids (arginine, leucine, valine and other essential amino acids) that can selectively modulate insulin sensitivity in the fasted or postprandial state.

Further studies are required to evaluate more de ply the effects of dietary proteins on insulin sensitivity and peripheral glucose utilization in rats by using the euglycemic clamp technique and the uptake of radiolabelled 2-deoxyglucose in individual insulin-sensitive tissues. The abundance and subcellular distribution of GLUT4 transporters should also be determined and related to the beneficial action of cod protein and soy protein isolates. Future studies should also assess the impact of dietary supplementation or depletion in arginine, leucine, valine or other essential amino acids on insulin sensitivity and glucose disposal in rats.

EXAMPLE 2:

We further investigated the benefits of fish protein in glucose utilization in high-fat fed rats.

Materials

2-deoxy-D-[1,2-^{3}H]glucose (2DG), and ^{14}C-sucrose was obtained from NEN (Boston, MA). Purified human insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN)

Treatment of animals

Male Wistar rats (200-250 g) purchased from Charles River (Montréal, Qc, Canada) were placed on a high-fat diet ad libitum for 4 weeks. The high-fat fed animals were sub-divided into 3 dietary groups fed cod, soy, or casein proteins. The composition of the purified diet is shown in Table 1. Diets were identical except for the amino acid composition of the dietary protein component which differed as detailed in Table 2. The cod protein was prepared in our laboratory by freeze-drying cod fillets, followed by a 24-h delipidation using diethyl ether as solvent in an Soxlet-type apparatus (Canadawide Scientific, Montréal, Qc, Canada). The energy content of the diet was measured in an automatic adiabatic calorimeter (Model 1241; Parr Instruments, Moline, IL) and found to be isoenergetic: casein (25.52 kJ/g), soy (25.25 kJ/g), cod (25.38 kJ/g). The protein content (N x 6.25) was assayed by Kjeldahl Foss autoanalyser (Model 1612; Foss Co., Hillerod, Denmark). The level of protein in the purified diets was adjusted to an isonitrogenous basis at the expense of carbohydrates. A control chow-fed group was treated and studied identically to those on the high-fat diets. This group was included in this study to assess the extent of insulin resistance induce by high-fat feeding. According to the manufacturer the chow diet contained, as percent of calories, 4.5% fat, 57.3% carbohydrate, and 18.1% protein and 14.3 kJ/g. Rats were housed in animal quarters maintained at 22 °C with a 12:12-h dark-light schedule. Food intake was analyzed daily. All experiments reported herein were approved by the Laval University Animal Care and Handling Committee.

Basal and euglycemic clamp studies.

Whole body insulin action was determined by the basal and hyperinsulinemic-euglycemic clamp procedure based on the method d scrib d by Roy et al (1998) (46).

Briefly, catheters were placed in the jugular vein and carotid artery (PE-10 polyethylene tubing, 0.28 mm ID, 0.61 mm OD) on anesth tiz d animals under isoflurane at least 4 days before performance of the clamp to allow recovery from the stress of surgery. Food intake was near normal postoperatively, and all rats were within 95% of operation weight on the day of study. The rats were fasted overnight, transferred to a quiet isolated room and weight. Unrestrained conscious animals were allowed to rest for 40 minutes before the initial blood sample (300 μL) was obtained. For hyperinsulinemic-euglycemic clamps a continuous intravenous infusion of insulin was then started at the rate of 4.0 mU $\cdot kg^{-1} \cdot min^{-1}$ and continued for 2 h with syringe pump (Razel, Stamford, CT) to achieve plasma insulin concentrations in the upper physiological range. Glucose solution was infused through the venous line at a variable rate to maintain blood glucose at the initial value. Blood samples (40μL) for measurement of plasma glucose were taken from the carotid artery catheter at 5-min intervals was monitored using glucometer Elite (Bayer, Etobicoke, ON). Every 20 min, an additional 300 μL of blood was withdrawn for determination of plasma insulin. To avoid dilution of the blood samples with blood from the dead space in the catheter, a volume of blood, 0.5 mL, equivalent to four times the dead space was drawn into a 1.0-mL syringe before each blood sampling. After the blood was drawn, erythrocytes from sample were suspended in saline and reinjected with the dead space blood. For determination of basal glucose uptake rates (see below), no infusion of insulin was given, and no exogenous glucose was necessary to maintain euglycemia.

In vivo 2-deoxy-D-glucose uptake.

Insulin action within individual tissues *in vivo* was studied as described previously (Roy et al 1998). Briefly, the nonmetabolizable glucose analogue 2,6-(^{3}H)-2-desoxy-D-glucose and D-(^{14}C)sucrose were administrated together in a intravenous bolus 120 min after the commencement of the insulin infusion. Blood samples for determination of plasma tracer concentrations were obtained at 5, 7.5, 10, 12.5, 15, 17.5 and 20 min after bolus administration for determination of radiolabeled 2DG and ^{14}C-sucrose. At the completion of the clamp rats were rapidly killed by decapitation and the following hindquarter muscles were rapidly removed and frozen into liquid nitrogen and stored at -80C for subsequent analysis: soleus (containing mainly slow-twitch oxidative fibers) , white (containing mainly fast-twitch glycolytic fibers) and red gastronemius (containing mainly fast-twitch oxidative-glycolytic fibers), white (containing mainly fast-twitch glycolytic fibers) and red tibialis (containing mainly fast-twitch oxidative-glycolytic fibers), and extensor digitorum longus (EDL, containing a mixture fast-twitch fibers) (47). The following tissue were rapidly removed from the trunk of the animal and frozen: heart, epiddymis and retroperitoneal white adipose fat pads, intrascapular brown fat and brain. All tissue samples (50-100 mg) were

dissolved in 1 mL of Solvable (NEN, Boston, MA) at 55 °C for 16 h. Thereafter, hydrogen peroxide (30% solution) was added to decreased quenching, followed by the addition of 8 mL of scintillation fluid (BCS, Amersham, Mississauga, ON)

Analytical determination

Plasma glucose determination was determined by the glucose oxidase method, using a Beckman glucose analyser (Beckman Instruments, Palo Alto, CA) Plasma insulin concentrations were measured by radioimmunoassay using a rat insulin specific kit from Linco (St. Louis, MO). ^{3}H and ^{14}C activity in an aliquot of plasma and of dissolved tissue samples were determined by liquid scintilation counter (Wallach 1409) using a dual-label counting program.

Statistics

The results are expressed as means ± SE. Multiple comparisons were made by analysis of variance followed by Duncan test for individual differences. The level of significance was $P<0.05$.

RESULTS

Physiological parameters

Table 6 shows energy intake, body weight, weight gain, and GWGER for the different dietary groups. These parameters were not different between the 3 dietary groups fed a high-fat diet. As expected, when compared to chow-fed rats, high-fat feeding increased energy intake, body weight, irrespective of the protein source. Body weight gain was not significantly different among the experimental groups and GWGER was lower in chow-fed than high-fat fed animals indicating that the experimental diets were nutritionally adequate to support normal growth. Weights of various muscles and adipose tissues were not significantly different among high-fat fed animal groups. However, high-fat fed animals showed increased (2-3-fold) gonadal and retroperitoneal fat weights as compared to chow-fed controls. Small increments (<10%) in the weights of some muscles (soleus, red tibialis) were also observed in high-fat fed rats. Fasting plasma glucose and insulin concentrations are shown in Table 8. Plasma glucose and plasma insulin levels did not differ between the 3 protein-fed groups but they were elevated as compared to chow-fed controls.

Whole-body glucose disposal during an hyperinsulinemic-euglycemic clamp

To evaluate the effect of casein, cod and soy proteins combined with high-fat diets on insulin action, we performed euglycemic clamps in which glucose was maintained constant and plasma insulin was kept at fasting levels (saline infusion) or raised to physiological post-prandial concentrations (983 ± 136 pM) by constant infusion of insulin (4 mU insulin /kg/min). As shown in Figure 7, the insulin-mediated glucose infusion rate (GIR) that was required to maintain plasma glucose level constant was ~40 % and ~60 % lower ($P<0.05$) in casein- and soy protein-fed groups, respectively, as compared to cod protein-fed high-fat fed animals. In the latter group,

the GIR was found to be similar to that observed in th chow-fed control group (dotted line). These data indicate that whole-body insulin action was impaired by high-fat feeding in rats fed cas in or soy protein, but that cod protein feeding prevented the development of insulin resistance in this model of insulin resistance.

Glucose uptake in individual tissues

To evaluate the effect of casein, cod and soy proteins on basal and insulin-stimulated glucose uptake in individual tissues of the high-fat fed animals, trace 2-deoxy-D-[1,2-^{3}H]-glucose (2DG) uptake was measured in a number of muscles, adipose tissues and brain. Basal (saline clamp) and insulin-stimulated 2DG uptake rates in seven different muscles are shown in Figures 8 and 9. Consistent with previous studies, four weeks of feeding a high-fat diet in casein-fed rats markedly decreased insulin-stimulated 2DG uptake in muscles enriched with either oxidative (type I), oxidative-glycolytic (type IIa) or glycolytic (type IIb) fibers, as compared to chow-fed animals (dotted line). Moreover, soy-fed rats did not show any detectable improvement in insulin action as compared to casein-fed animals. In stack contrast, high-fat fed rats also fed cod protein showed increased insulin action as compared to soy or casein fed groups, and exhibited comparable rates of 2DG uptake than chow-fed controls. Similar results were observed in cardiac muscle (Figure 10). Basal 2DG uptakes in muscles were not different among high-fat fed groups (Figures 8 and 9) and no effects of high-fat feeding were observed on basal 2DG uptake rates in any of the muscles as compared to chow-fed animals (data not shown). Thus, these results demonstrate that cod feeding prevents the development of insulin resistance in skeletal muscles of high-fat fed rats. These data are in accordance with the restoration of a normal whole-body insulin action on glucose disposal (Fig. 7) since muscles are the primary sites of glucose utilization in the insulin-stimulated state.

We also examined the effects of the dietary protein treatments on insulin-stimulated 2DG uptake in brown (Figure 10) and white adipose tissues (Figure 11). It should be noted that glucose uptake values were much greater in brown adipose tissue than white adipose tissues whatever the diet consumed (cf y axes in Fig 6 vs 10), in accordance with previous studies (46). High-fat feeding was associated with impaired insulin action in both casein and soy-protein fed rats, as compared to chow-fed controls. Cod protein fed animals showed an improved insulin-mediated glucose disposal, but it failed to reach the level of significance as compared to the other dietary groups. However, no effects of the dietary proteins were observed in white fat depots (Figure 6). As compared to chow-fed controls, insulin-mediated 2DG uptake was markedly reduced in all high-fat fed groups. No effects of any treatments on basal 2DG uptake were observed.

Figure 7 shows 2DG uptake in brain, an insulin-insensitive tissue used here for comparison purposes. No effects of insulin and/or the dietary proteins were observed

in brain. Moreover, high-fat feeding did not significantly affect 2DG uptake in this tissue.

EXAMPLE 3:

High-fat feeding impairs insulin-stimulated glucose transport and GLUT4 translocation in rodents. However, we found that feeding fish (cod) protein, but not soy or casein proteins, prevents the development of insulin resistance in skeletal muscles of high-fat fed rats. We have tested the hypothesis that the beneficial effect of fish protein on insulin action could be explained by changes in GLUT4 expression and/or GLUT4 translocation to the muscle cell surface. Male Wistar rats were fed for 4 weeks a high-fat diet (65 % energy from fat, 20 % from carbohydrate, 15 % from protein) in which the protein source was either cod, soy, or casein. After the dietary treatments, hindlimb muscles enriched with different fiber types were removed and GLUT4 expression was evaluated. GLUT4 mRNA levels were greater in soleus (type I) and red tibialis (type IIa) muscles from cod-fed rats as compared to soy-fed animals (1.6-1.8-fold, $p<0.05$). However, no differences in GLUT4 transcript levels were observed between rats fed cod or casein proteins. GLUT4 mRNA was not regulated by dietary proteins in white gastrocnemius (type IIb). On the other hand, GLUT4 protein levels in muscle homogenates were not significantly different among dietary groups for any given muscle. We therefore examine the effects of the dietary proteins on insulin-stimulated GLUT4 translocation to the muscle cell surface of high-fat fed rats. Rats from all groups were subjected to a physiological hyperinsulinemia through the infusion of insulin (4 mUminkg) or saline (controls) during an euglycemic clamp and GLUT4 recruitment to the plasma membrane (PM) and the T-Tubules (TT) was determined by subcellular fractionation of mixed hindlimb muscles. Insulin was found to stimulate the translocation of GLUT4 to both the PM and TT (1.7-1.8-fold, $p<0.05$) in rats fed cod protein. In marked contrast, insulin failed to increase GLUT4 at the cell surface of rats fed either casein or soy proteins. These results indicate that fish protein prevents the development of insulin resistance in muscle of high-fat fed rats at least in part by restoring insulin-stimulated GLUT4 translocation to the cell surface.

Although the present invention has been described hereinabove by way of a preferred embodiment thereof, this embodiment can be modified at will, within the scope of the appended claims, without departing from the spirit and nature of the subject invention.

Table 1. Composition of the purified diets in Example 1

Ingredients	Casein (g/100g)	Soy protein	Cod protein
Casein	22.78	-	-
Soy protein	-	22.90	-
Cod protein	-	-	21.87
Sucrose	55.52	55.40	56.43
Coconut oil	10	10	10
Corn oil	1	1	1
Cholesterol	1	1	1
Cellulose	5	5	5
Vitamin mix	1	1	1
Mineral mix	3.5	3.5	3.5
Choline bitartrate	0.2	0.2	0.2

	% energy density
Carbohydrate	55
Protein	20
Fat	25

Table 2. Amino acid composition of protein sources[1]

	Casein	Cod protein	Soy protein
Alanine	3.40	6.74	4.49
Arginine	3.23	6.29	7.45
Aspartic acid	7.99	11.14	11.48
Glutamic acid	20.12	16.75	18.79
Glycine	2.09	5.39	4.33
Histidine	2.83	2.27	2.94
Isoleucine	3.32	3.24	3.68
Leucine	8.75	8.31	7.93
Methionine	2.04	1.98	0.66
Lysine	7.76	9.41	6.60
Phenylalanine	5.25	4.22	5.51
Proline	10.72	4.42	5.62
Serine	6.12	5.55	6.03
Threonine	4.29	4.84	4.27
Tyrosine	5.76	4.31	3.95
Valine	4.52	3.86	3.96
BAA[2]	16.59	15.41	15.57
EAA[3]	35.93	35.86	32.61

[1] Means of three determinations; in g of amino acid per 100 g of amino acids

[2] Sum of branched-chain amino acids: leucine, isoleucine and valine.

[3] Sum of essential amino acids except histidine.

Table 3. Food intake, weight gain and food intake during the test meal of rats f d the purified diets for experiment 1 (IVGTT) and experiment 2 (Test meal)[1] of Exampl 1

Dietary Group(n)	Food intake (g/day/animal)	Weight gain (g/day/animal)	Food intake last m al (g/animal)
Experiment 1 (IVGTT)			
Casein (9)	20.4±0.7[a]	5.0±0.3[a]	NA
Cod protein (7)	19.9±0.6[a]	5.0±0.4[a]	
Soy protein (7)	20.9±0.8[a]	4.6±0.3[a]	
Experiment 2 (Test meal)			
Casein (7)	21.4±0.6[a]	5.8±0.2[a]	3.8±0.8[a]
Cod protein (10)	20.0±0.5[a]	5.5±0.3[a]	3.7±0.4[a]
Soy protein (9)	21.8±0.8[a]	5.6±0.3[a]	3.8±0.8[a]

[1]Values are means±SEM. [a] Values bearing same superscript were not significantly different ($p<0.05$) according to Duncan multiple range test.

Table 4. Plasma amino acid concentrations in fasted rats (μmol/L)

	Casein	Cod protein	Soy protein
Alanine	326.26 ± 19.39 [a]	301.52 ± 15.12 [a]	305.88 ± 11.77 [a]
Arginine	182.94 ± 23.65 [a]	147.05 ± 14.99 [a]	170.11 ± 6.69 [a]
Aspartic acid	16.59 ± 2.73 [b]	14.79 ± 1.17 [b]	21.65 ± 2.28 [a]
Citruline	78.57 ± 12.60 [a]	54.46 ± 4.18 [b]	54.26 ± 4.89 [b]
Glutamine	1397.50 ± 229.83 [a]	1060.12 ± 152.02 [b]	1129.18 ± 105.40 [a]
Glycine	231.29 ± 15.37 [b]	268.54 ± 17.80 [b]	342.93 ± 37.70 [a]
Histidine	69.51 ± 5.78 [a]	53.86 ± 6.46 [b]	74.78 ± 5.47 [a]
Leucine	118.59 ± 9.25 [a]	105.65 ± 6.86 [a]	115.72 ± 7.04 [a]
Lysine	398.28 ± 23.20 [a]	391.50 ± 28.15 [a]	383.43 ± 14.08 [a]
Methionine	64.71 ± 7.65 [a]	62.51 ± 9.68 [a]	68.98 ± 6.89 [a]
Proline	158.92 ± 5.91 [a]	134.83 ± 8.64 [a]	170.38 ± 13.25 [a]
Serine	225.75 ± 17.21 [ab]	206.42 ± 10.61 [b]	266.25 ± 13.33 [a]
Taurine	94.76 ± 6.51 [b]	125.28 ± 10.51 [a]	71.71 ± 7.88 [c]
Tyrosine	68.53 ± 8.41 [a]	64.55 ± 4.90 [a]	76.07 ± 2.98 [a]
Threonine	300.27 ± 25.38 [a]	221.79 ± 16.10 [b]	267.86 ± 12.32 [ab]
Valine	162.52 ± 14.27 [a]	130.66 ± 8.28 [a]	151.88 ± 10.60 [a]
LAR [1]	2.33 ± 0.25 [a]	2.76 ± 0.20 [a]	2.27 ± 0.10 [a]
BAA [2]	355.95 ± 24.45 [a]	302.36 ± 20.43 [a]	344.22 ± 21.24 [a]
EAA [3]	1189.78 ± 64.36 [a]	1046.87 ± 62.54 [a]	1139.62 ± 36.65 [a]
TAA [4]	4273.82 ± 194.54 [a]	4120.54 ± 161.30 [a]	4335.57 ± 108.62 [a]

Values are expressed as means ± SEM. Groups bearing a similar superscript are not significantly different ($p<0.05$).

[1] LAR : Lysine arginine ratio.

[2] BAA : Sum of branched-chain amino acids: leucine, isoleucine and valine.

[3] EAA : Sum of essential amino acids except histidine.

[4] TAA : Sum of total amino acids.

Table 5. Composition of the purified diets in Example 2

Ingredients	Casein	Soy protein isolate (g/100g)	Cod protein
Casein[a]	22.55	-	-
Soy protein isolate[b]	-	22.81	-
Cod protein[c]	-	-	21.47
Sucrose[d]	24.55	24.29	25.56
Lard[d]	19.8	19.8	19.8
Corn oil[e]	19.8	19.8	19.8
Cellulose[g]	5	5	5
Vitamin mix[h]	1.4	1.4	1.4
Mineral mix[i]	6.7	6.7	6.7
Choline bitartrate[d]	0.2	0.2	0.2
BHT[d]	0.004	0.004	0.004

[a] Highly purified casein, ICN Biochemicals, Cleveland, OH; 88% protein, 0.1% lipid.

[b] Soy protein isolate, ICN Nutritional Biochemicals; 87% protein, 0.3% lipid.

[c] Cod protein, prepared in our laboratory; 91% protein, 0.2% lipid.

[d] ICN Nutritional Biochemicals.

[e] Mazola corn oil, Best Foods, Canada Starch, Montréal, Québec, Canada.

[g] Alphacel nonnutritive bulk, ICN Nutritional Biochemicals.

[h] Vitamin mix, Teklad Test Diets, Madison, WI.

[i] Mineral Mix AIN-76, ICN Nutritional Biochemicals.

Table 6. Body weight , energy intake, weight gain, and gross weight gain efficiency ratio (GWGE) of the diff rent dietary groups.[1] of Example 3

Dietary group	(n)	Body weight (g)	Energy intake (KJ/day/animal)	Weight gain (g/day/animal)	GWGER [2]
Casein	(9)	380 ± 20	373 ± 18	3.2 ± 0.4	0.22 ± 0.02
Cod protein	(7)	400 ± 21	378 ± 20	3.6 ± 0.2	0.24 ± 0.02
Soy protein	(7)	368 ± 23	363 ± 12	3.0 ± 0.4	0.21 ± 0.02
Chow	(7)	351 ± 20	320 ± 12	3.1 ± 0.4	0.09 ± 0.02

[1] Values are means ± SEM.

[2] GWGE= weight gain(g/day/animal)/food intake(g/day/animal)

Table 7. Tissue weights (g) of experimental dietary groups after the hyperinsulinemic euglycemic clamp.

	Casein	Cod protein	Soy protein	Chow
Heart	1.17 ± 0.03 [a]	1.11 ± 0.03 [a]	1.14 ± 0.04 [a]	1.06 ± 0.05 [a]
Soleus	0.20 ± 0.01 [a]	0.20 ± 0.01 [a]	0.19 ± 0.01 [a]	0.18 ± 0.01 [a]
White gastrocnemius	0.20 ± 0.02 [a]	0.20 ± 0.03 [a]	0.19 ± 0.02 [a]	0.22 ± 0.02 [a]
Red gastrocnemius	0.19 ± 0.02 [a]	0.19 ± 0.02 [a]	0.18 ± 0.01 [a]	0.18 ± 0.02 [a]
White tibialis	0.45 ± 0.02 [a]	0.46 ± 0.02 [a]	0.42 ± 0.02 [a]	0.44 ± 0.02 [a]
Red tibialis	0.25 ± 0.01 [a]	0.26 ± 0.02 [a]	0.23 ± 0.02 [a]	0.24 ± 0.01 [a]
EDL	0.17 ± 0.01 [a]	0.17 ± 0.01 [a]	0.16 ± 0.01 [a]	0.16 ± 0.01 [a]
Quadricep	2.51 ± 0.12 [a]	2.70 ± 0.10 [a]	2.20 ± 0.08 [a]	2.23 ± 0.06 [a]
White gonadal	3.33 ± 0.30 [a]	3.86 ± 0.40 [a]	3.23 ± 0.42 [a]	1.92 ± 0.10 [b]
White retroperitoneal	2.67 ± 0.35 [a]	3.22 ± 0.50 [a]	2.88 ± 0.44 [a]	1.17 ± 0.15 [b]
Brown adipose tissue	0.29 ± 0. 03 [a]	0.29 ± 0.02 [a]	0.33 ± 0.04 [a]	0.12 ± 0.03 [b]
Brain	1.49 ± 0.06 [a]	1.51 ± 0.07 [a]	1.52 ± 0.07 [a]	1.42 ± 0.04 [a]

Means ± SEM , n= 7-9 animals per groups. Within a column, values followed by a same superscript letter are not significantly different.

Table 8. Fasting plasma glucose and insulin of rats fed the purified diets.[1]

Dietary Group (n)	Glucose (mmol/L)	Insulin (pmol/L)
Casein (16)	5.7±0.2	238±42
Cod protein (14)	6.2±0.4	294±32
Soy protein (15)	5.3±0.2	299±70
Chow (12)	5.0± 0.2	202 ± 51

[1] Values are means ± SE.

Table 9. Nitrite and nitrate levels in fasted rats fed either diets during 28 days(nM).

	High-sucrose diets	High-fat diets
casein	4413 ± 363[b]	1960 ± 216[b]
cod protein	6902 ± 423[a]	3819 ± 1832[a]
my protein	7828 ± 1515[a]	3807 ± 415[a]

Values are means + SEM from 7 to 10 animals in each experimental groups. Within a column, values followed by a same superscript letter are not significantly different ($P<0.05$).

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What is claimed is:

1. The use of fish protein, a peptide or an amino acid thereof, or of a derivative therefrom, to restore a normal insulin function in an insulin-resistant mammal.

2. The use of fish protein, a peptide or an amino acid thereof, or of a derivative therefrom, in the making of a medication to restore a normal insulin function in an insulin-resistant mammal.

3. The use as defined in claim 1 or 2, wherein said mammal suffers of insulin-resistance or diabetes.

4. The use as defined in claim 1 or 2, wherein said mammal suffers of obesity complications.

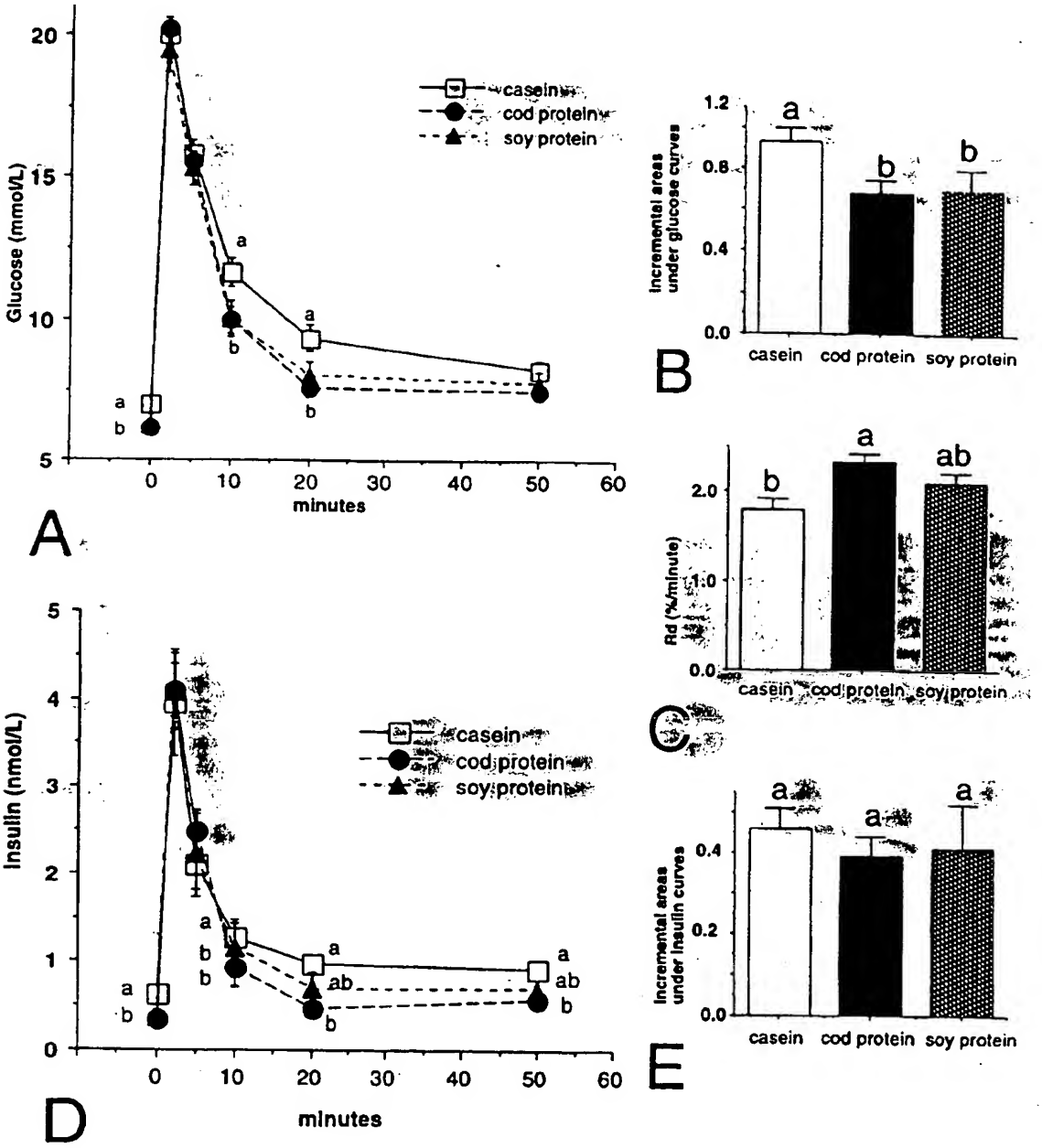

Glucose (mmol/L)
minutes
casein
cod protein
soy protein
A
Incremental areas under glucose curves
casein
cod protein
soy protein
B
Rd (%/minute)
casein
cod protein
soy protein
C
Insulin (nmol/L)
minutes
casein
cod protein
soy protein
D
Incremental areas under insulin curves
casein
cod protein
soy protein
E

FIGURE 1

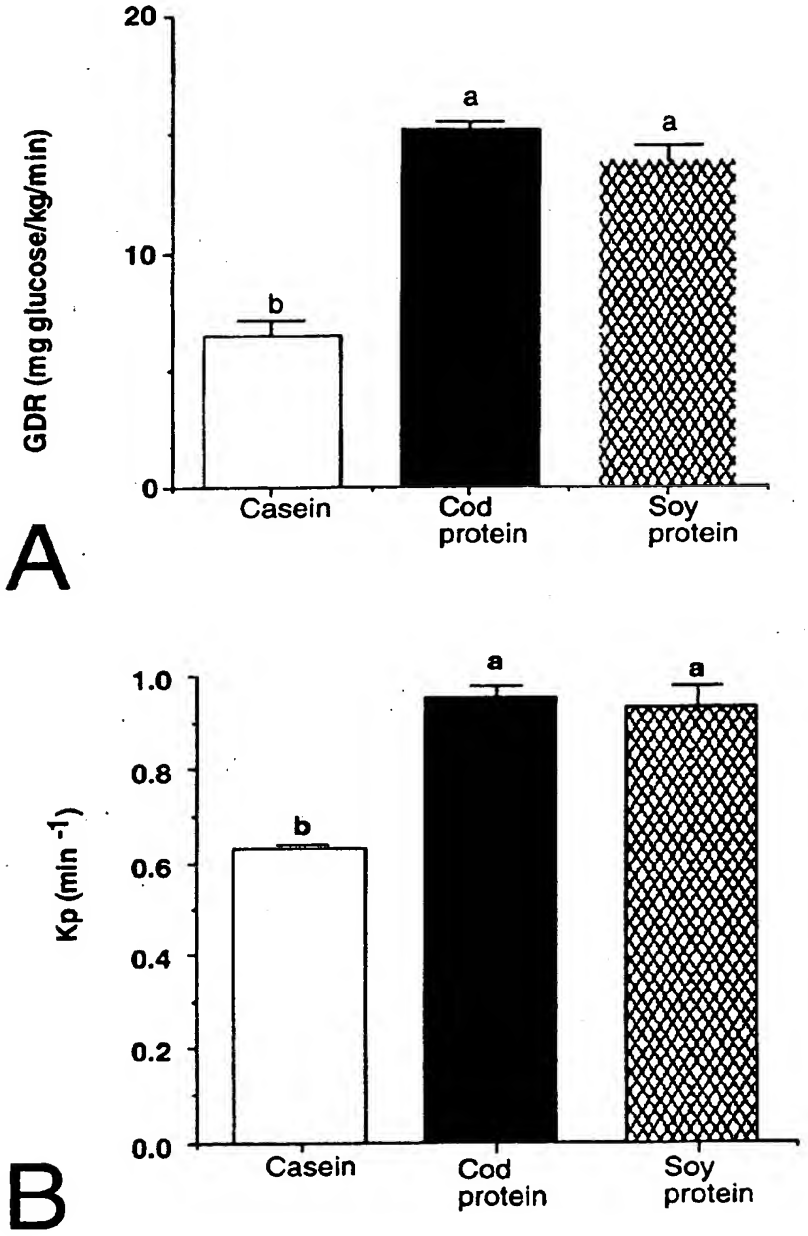

20
10
0
GDR (mg glucose/kg/min)
a
a
b
Casein
Cod protein
Soy protein
A
1.0
0.8
0.6
0.4
0.2
0.0
Kp (min -1)
a
a
b
Casein
Cod protein
Soy protein
B

FIGURE 2

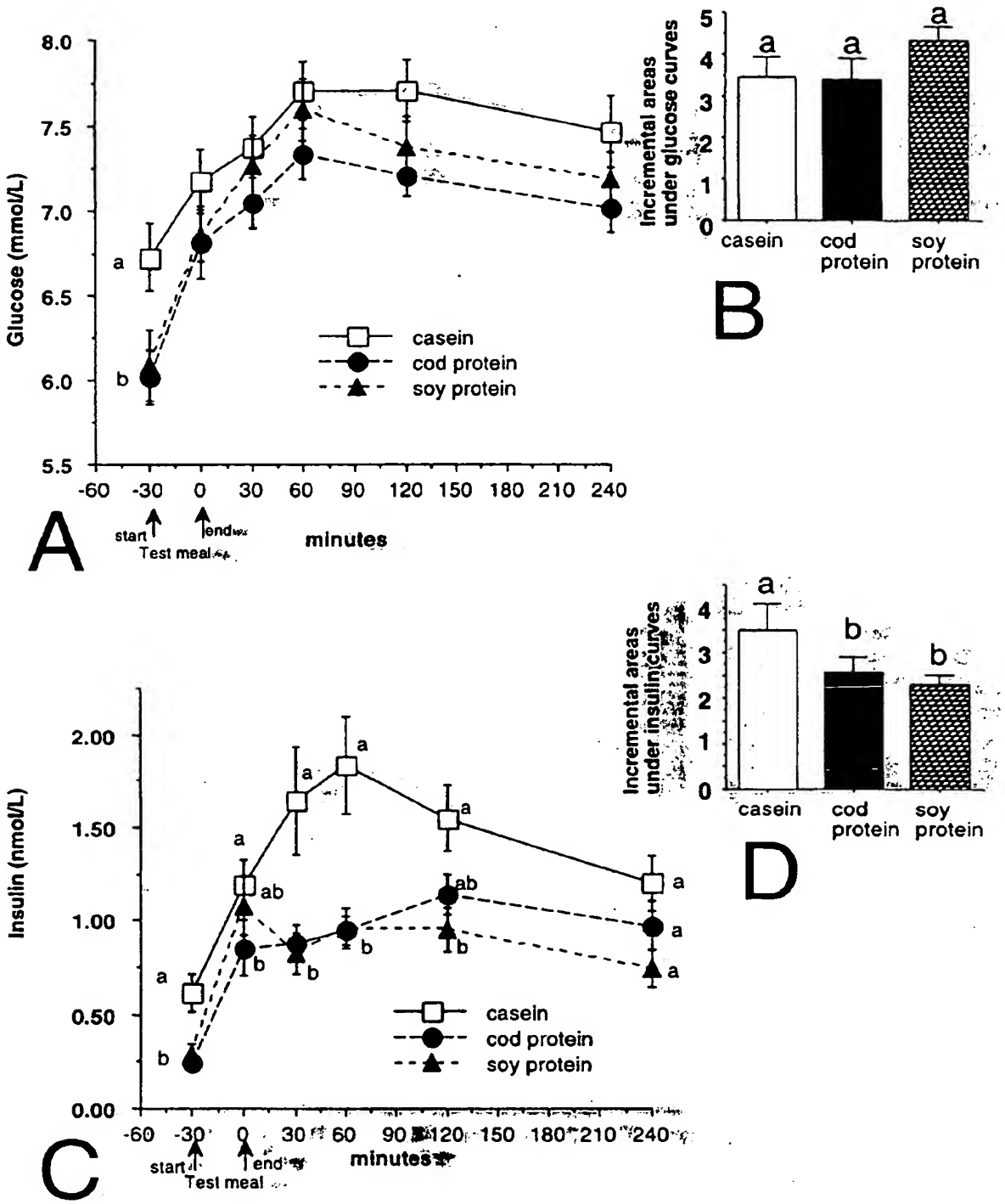

Glucose (mmol/L)
8.0
7.5
7.0
6.5
6.0
5.5
-60 -30 0 30 60 90 120 150 180 210 240
start
end
Test meal
minutes
casein
cod protein
soy protein
A
Incremental areas under glucose curves
5
4
3
2
1
0
casein
cod protein
soy protein
B
Insulin (nmol/L)
2.00
1.50
1.00
0.50
0.00
-60 -30 0 30 60 90 120 150 180 210 240
start
end
Test meal
minutes
casein
cod protein
soy protein
C
Incremental areas under insulin curves
4
3
2
1
0
casein
cod protein
soy protein
D

FIGURE 3

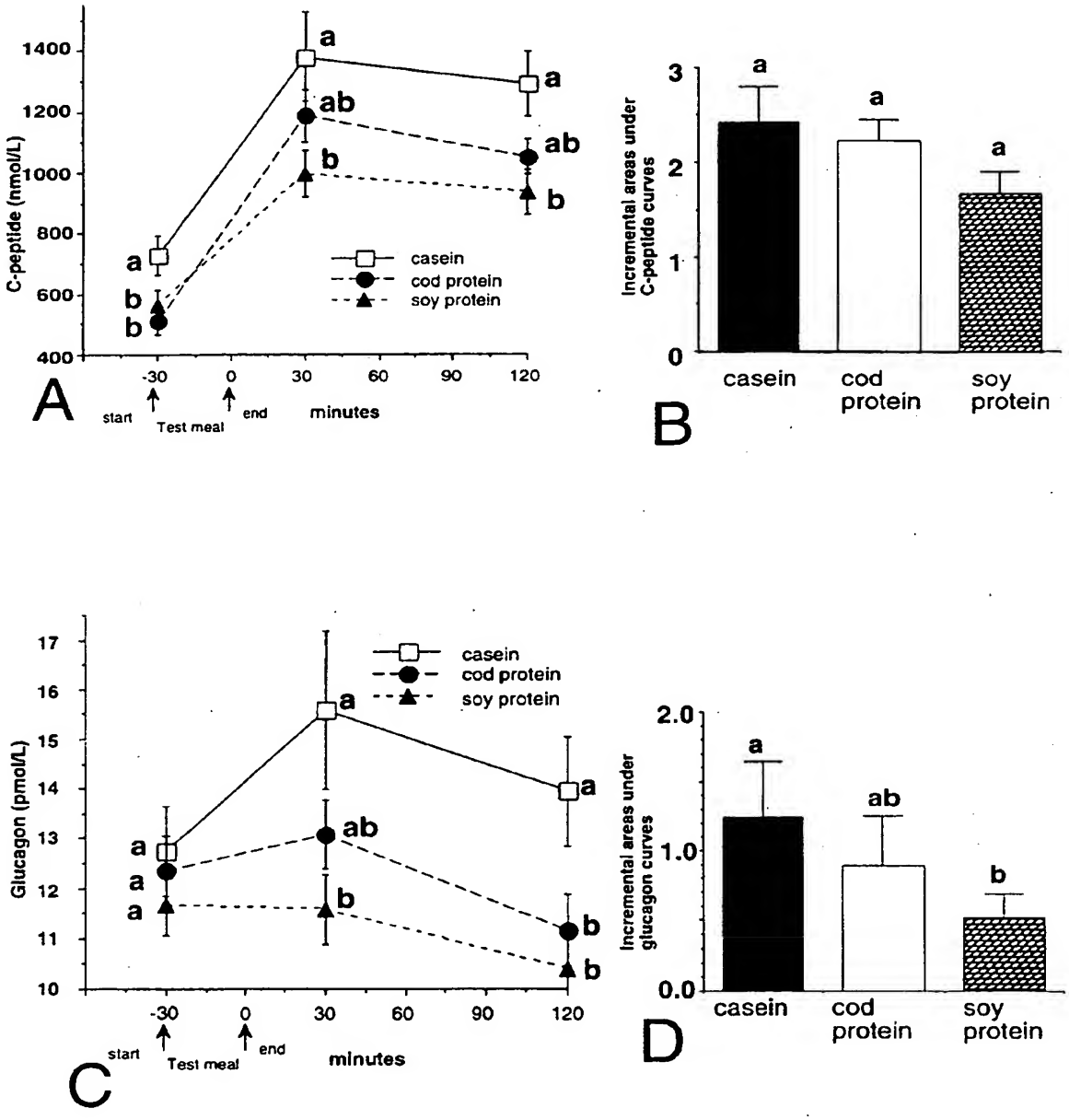
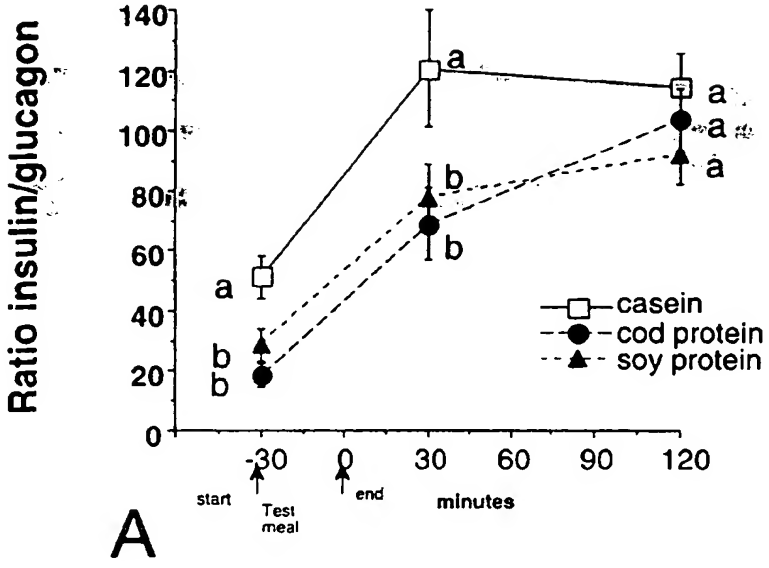
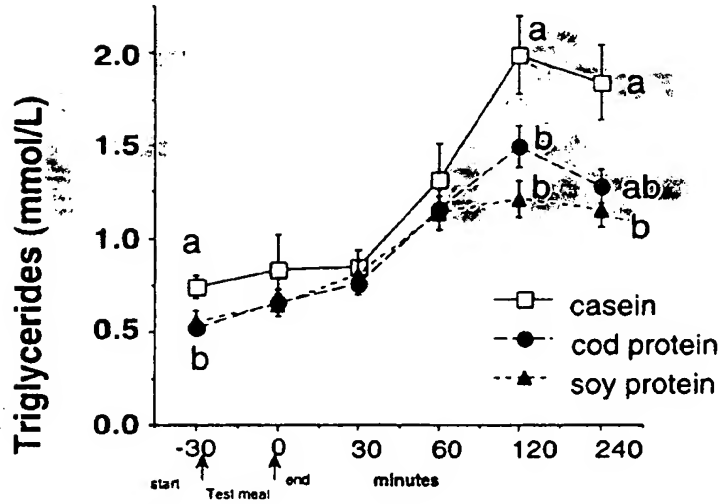

C-peptide (nmol/L)
1400
1200
1000
800
600
400
a
ab
b
a
ab
b
a
b
b
casein
cod protein
soy protein
-30
0
30
60
90
120
start
Test meal
end
minutes
A
Incremental areas under C-peptide curves
3
2
1
0
a
a
a
casein
cod protein
soy protein
B
Glucagon (pmol/L)
17
16
15
14
13
12
11
10
a
a
a
a
ab
b
b
b
casein
cod protein
soy protein
-30
0
30
60
90
120
start
Test meal
end
minutes
C
Incremental areas under glucagon curves
2.0
1.0
0.0
a
ab
b
casein
cod protein
soy protein
D

FIGURE 4


Ratio insulin/glucagon
140
120
100
80
60
40
20
0
a
a
a
b
b
b
b
casein
cod protein
soy protein
-30
0
30
60
90
120
start
Test meal
end
minutes

A


Triglycerides (mmol/L)
2.0
1.5
1.0
0.5
0.0
a
a
b
b
ab
b
casein
cod protein
soy protein
-30
0
30
60
120
240
start
Test meal
end
minutes

B

FIGURE 5

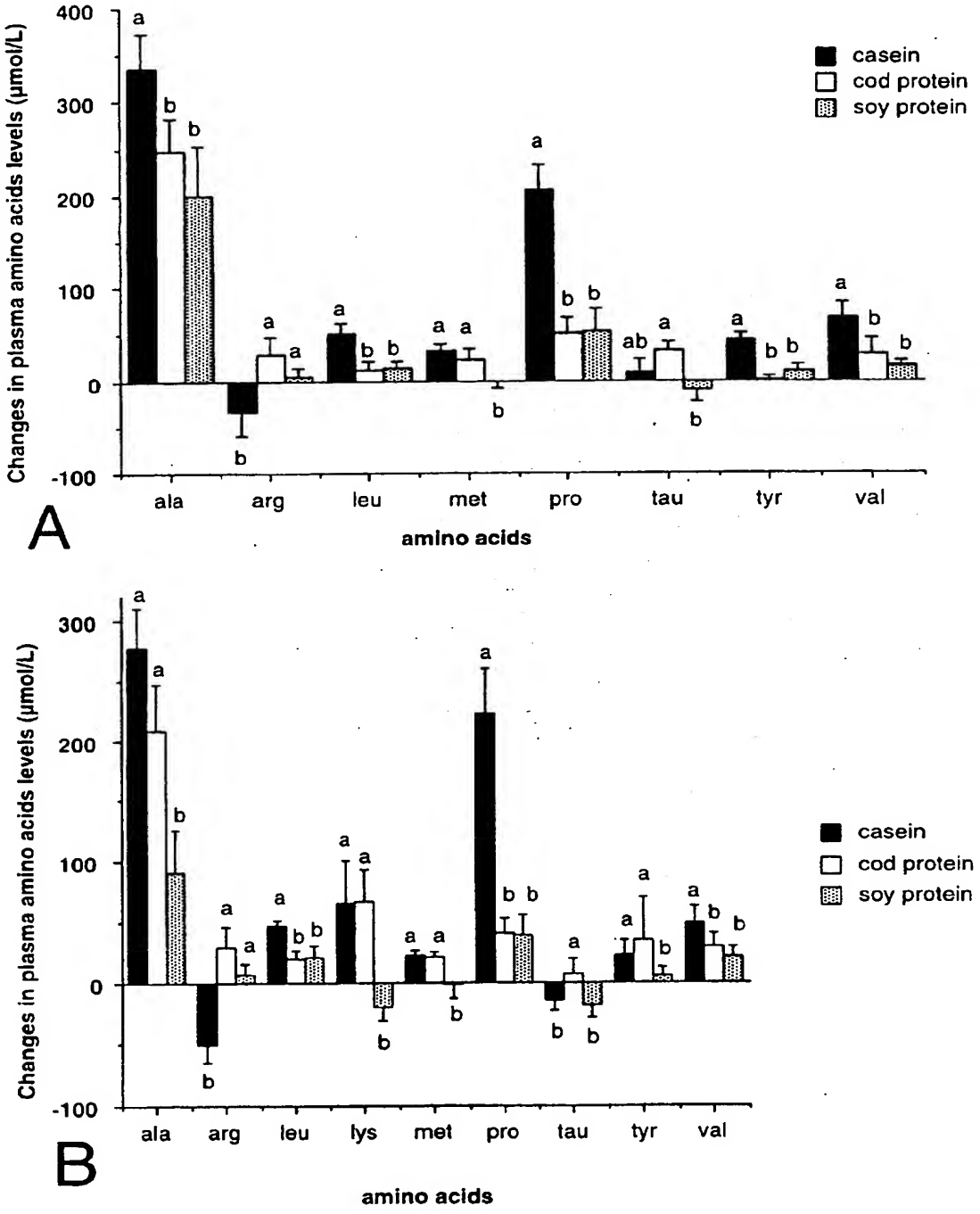

Changes in plasma amino acids levels (µmol/L)
400
300
200
100
0
-100
casein
cod protein
soy protein
ala
arg
leu
met
pro
tau
tyr
val
amino acids
A
Changes in plasma amino acids levels (µmol/L)
300
200
100
0
-100
casein
cod protein
soy protein
ala
arg
leu
lys
met
pro
tau
tyr
val
amino acids
B

FIGURE 6

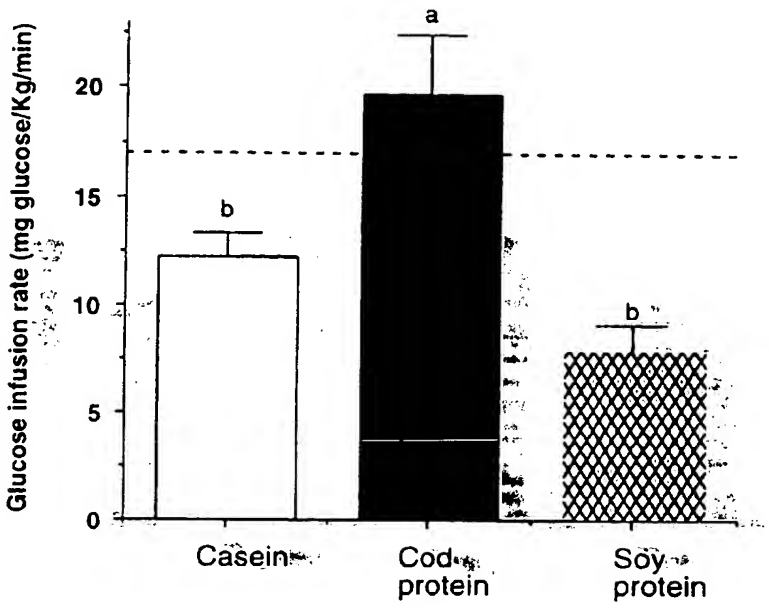

Glucose infusion rate (mg glucose/Kg/min)
0
5
10
15
20
a
b
b
Casein
Cod protein
Soy protein

FIGURE 7

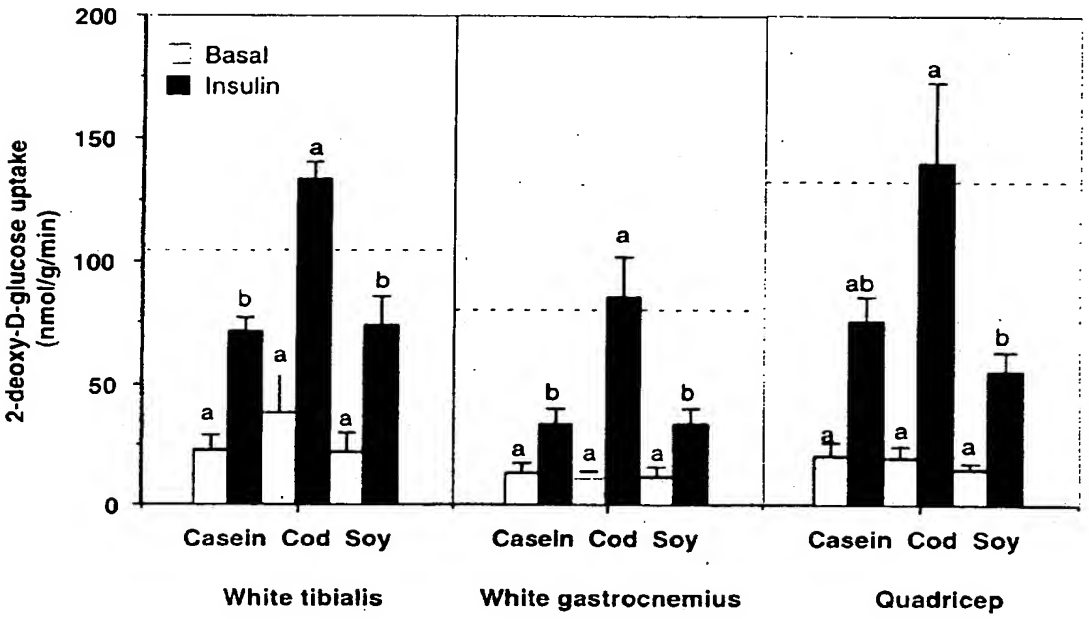

Basal
Insulin
2-deoxy-D-glucose uptake (nmol/g/min)
200
150
100
50
0
a
b
a
a
b
a
a
b
a
a
b
a
a
ab
a
a
b
a
Casein Cod Soy
Casein Cod Soy
Casein Cod Soy
White tibialis
White gastrocnemius
Quadricep

FIGURE 8

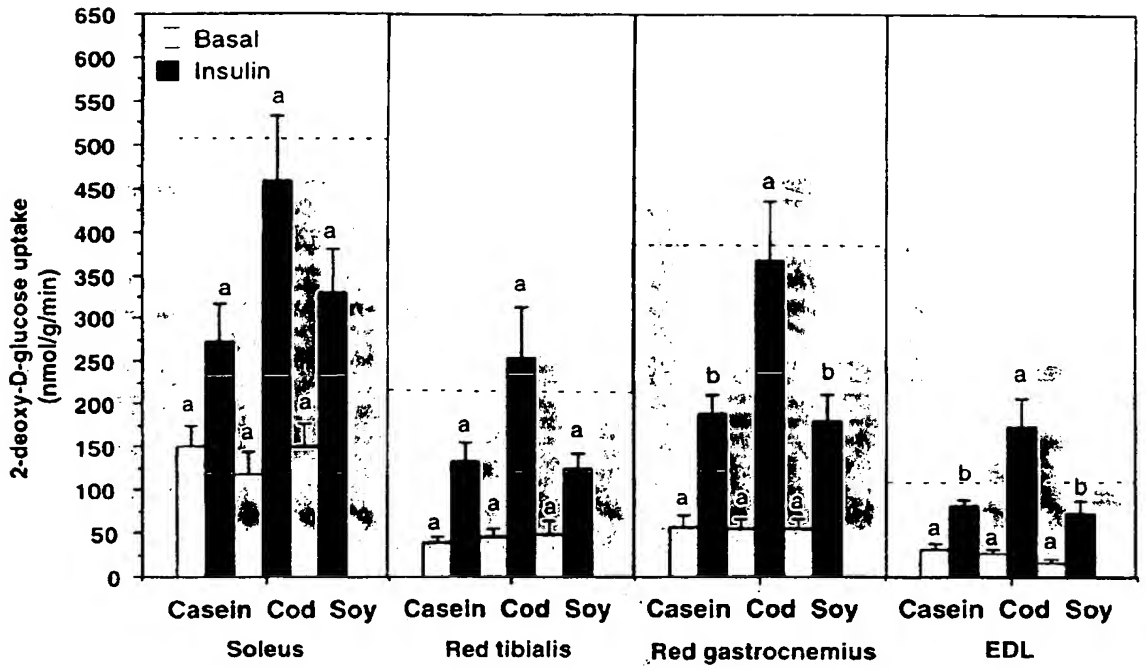

Basal
Insulin
2-deoxy-D-glucose uptake (nmol/g/min)
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Casein Cod Soy
Soleus
Casein Cod Soy
Red tibialis
Casein Cod Soy
Red gastrocnemius
Casein Cod Soy
EDL

FIGURE 9

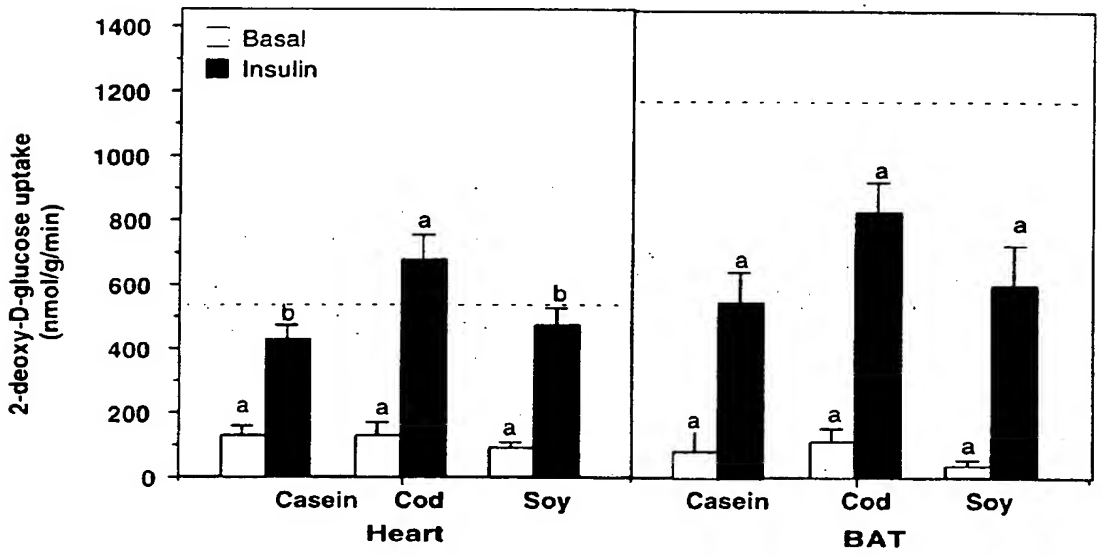

Basal
Insulin
2-deoxy-D-glucose uptake (nmol/g/min)
1400
1200
1000
800
600
400
200
0
a
b
a
a
a
b
a
a
a
a
a
a
Casein
Cod
Soy
Heart
Casein
Cod
Soy
BAT

FIGURE 10

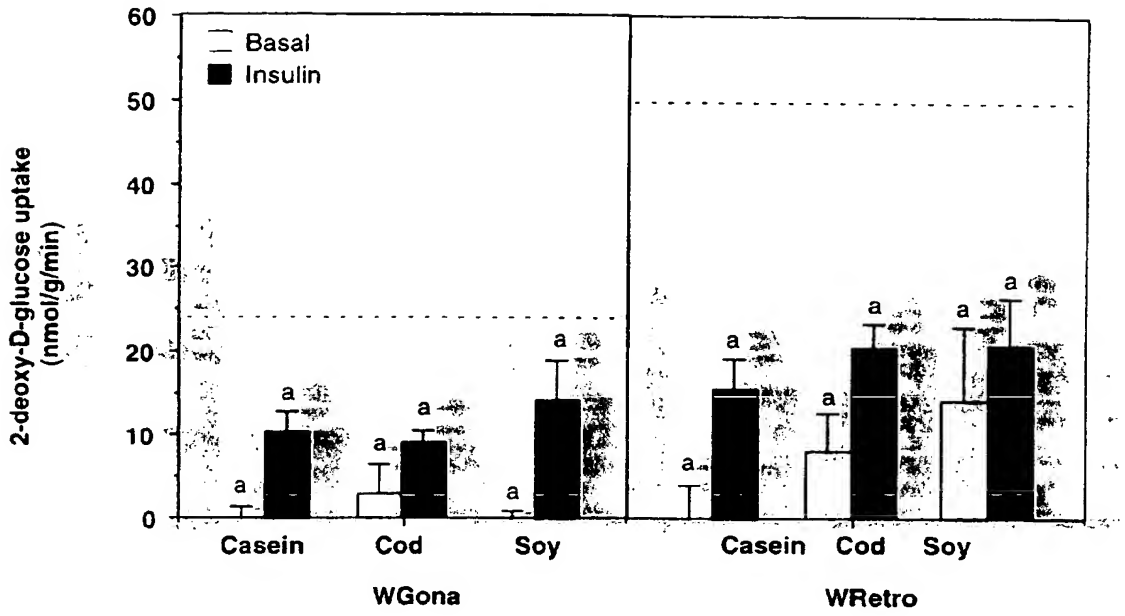

60
50
40
30
20
10
0
Basal
Insulin
2-deoxy-D-glucose uptake
(nmol/g/min)
a
Casein
Cod
Soy
WGona
WRetro

FIGURE 11

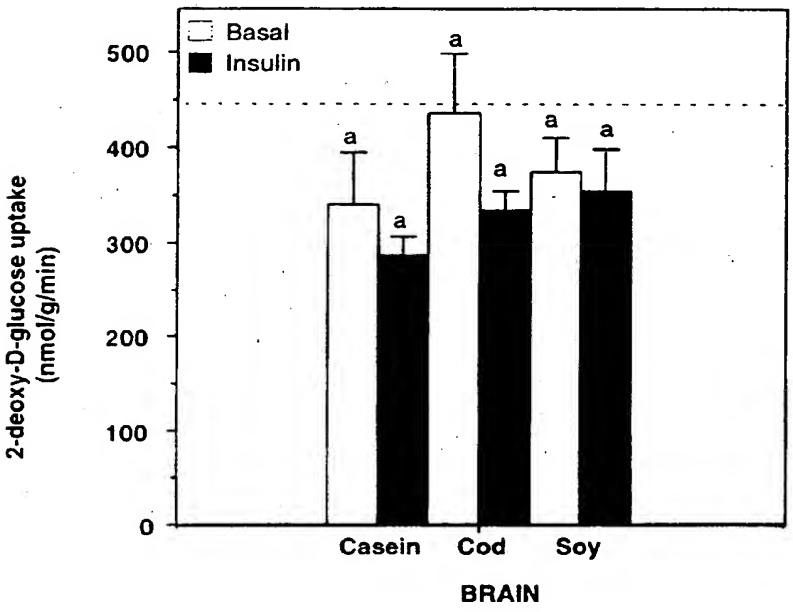

Basal
Insulin
2-deoxy-D-glucose uptake
(nmol/g/min)
500
400
300
200
100
0
a
a
a
a
a
a
Casein
Cod
Soy
BRAIN

FIGURE 12